High Resolution and High Precision Analysis of Barbiturates and Metabolite in Human Body Fluids Using a Monolithic Spin Tip and UPLC-Q-ToF-MS

Yukiko SHOUJI[1)], Chika HASEGAWA[*1, 2)], Xiao-Pen LEE[*1)], Masaya FUJISHIRO[1)], Takaaki MATSUYAMA[1)], Miho YAMADA[1)], Yuka KATO[1)], Noriko NEMOTO[1)], Takeshi KUMAZAWA[1, 3)], Shinichi SUZUKI[1, 4)], and Keizo SATO[1)]

[1)] *Department of Legal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan.*

[2)] *Department of Legal Medicine, Toho University School of Medicine.*

[3)] *Seirei Christopher University School of Nursing.*

[4)] *Identification Center, National Research Institute of Police Science.*

[*] To whom corresponding should be addressed.

Running title: High resolution and high precision analysis of barbiturates

Abstract

A high-throughput method was developed for determination of five barbiturates (phenobarbital, cyclobarbital, amobarbital, secobarbital, and thiopental) and a metabolite in human body samples using a new Monolithic C_{18} gel-packed Spin Tip and ultra-performance liquid chromatography (UPLC)-quadrupole-time of flight (Q-ToF) mass spectrometry (MS). Plasma (20 µl) or urine (100 µl) spiked with the five barbiturates and 5-(4-methylphenyl)-5-phenylhydantoin (internal standard, IS) were mixed with distilled water. The mixture was extracted with Monolithic C_{18} Spin Tip. The analytes retained on the C_{18} phase were then eluted with methanol. The eluate was injected directly into analytical column (Waters Acquity BEH C_{18}, 50 mm × 2.1 mm i.d., particle size 1.7 µm). Quantification was performed by multiple reaction monitoring with negative-ion electrospray ionization (ESI). Good separation and clear peak shapes of the five drugs were achieved within an analysis time of 6 min, including the extraction time. All drugs spiked in plasma showed recoveries of 86–98%. The regression equations for the five drugs showed excellent linearities in the range of 5–500 ng/20 µl of plasma, and the limits of detection and i-Fit was 1 ng/20 µl. The method was successfully applied to determine the level of amobarbital and its metabolite in human plasma

and urine, respectively, after oral administration to a volunteer. This new method can lead to a wide range of applications in quantitative determination of drugs and metabolites in the clinical and forensic fields.

Keywords Barbiturate drugs, Monolithic C_{18} Spin Tip, UPLC-Q-ToF-MS

Introduction

Barbiturates are a class of drugs derived from barbituric acid that act as sedative-hypnotics to the central nervous system [1, 2]. These drugs are frequently encountered in emergency toxicology screening, drug abuse testing, and forensic medical examinations [3, 4]. Methods for determining the concentration of barbiturates in human samples are required for diagnosis and effective treatment of intoxication and for forensic purposes.

Several methods have been reported for the determination of the levels of barbiturates in various matrices using gas chromatography [5], gas chromatography-mass spectrometry [6, 7], high-performance liquid chromatography (HPLC) [8, 9], and HPLC-mass spectrometry (MS) or HPLC-tandem MS (MS-MS) [10, 11]. Most of these techniques employ extraction methods such as liquid-liquid extraction (LLE) [6, 8-10], solid-phase extraction (SPE) [11], or solid-phase microextraction [7] to remove impurities contained in human body fluid samples. Although LLE and SPE can successfully extract drugs from biological fluids, these procedures are generally performed in the off-line mode and are often labor-intensive and time-consuming.

In this study, we present and discuss new and unique monolithic silica SPE techniques that use spin tips. An advantage of Monolithic C_{18} gel-

packed SPE Spin Tip for sample preparation is that extraction can be carried out more easily and rapidly than with the conventional SPE cartridges (Fig 1). The small bed volume and sorbent mass within the monolithic spin tip allow for the use of a reduced solvent volume, smaller elution volume, reduced time for the extraction step, and higher throughput.

Currently, HPLC-MS/MS mainly based on triple quadrupole instruments, have been widely applied for the analysis of biological samples [10-17]. When using HPLC-MS/MS operating in multiple reaction monitoring (MRM) mode, although quantitative information can be obtained, the qualitative information needed to support the structural elucidation of analytes is poor. Recently, HPLC/quadrupole time-of-flight (Q-TOF)-MS has been used for the unequivocal confirmation of the compounds from biological and environment samples by accurate mass measurement of protonated, and deprotonated molecules [18-21]. Accurate mass measurements of MS/MS product ions have also become particularly important in the structural elucidation of unknowns. Q-ToF-MS is unique in its ability to give accurate mass measurements of product ions, assuring the correct identification of target compounds and unknowns.

In the present study, we established a recommendable procedure for analyzing five barbiturates (phenobarbital, cyclobarbital, amobarbital, secobarbital, thiopental) from human plasma samples and the metabolite of

amobarbital from human urine using the Monolithic C_{18} Spin Tip and UPLC-Q-ToF-MS analysis. This is the first report on use Monolithic C_{18} Spin Tip for the extraction of barbituric acid drugs from human body fluids.

Materials and methods

Materials

Amobarbital was provided by Nippon Shinyaku Co. Ltd. (Kyoto, Japan). Cyclobarbital was purchased from Tokyo Kasei Industry Co. Ltd. (Tokyo, Japan). Phenobarbital, secobarbital, thiopental, and 5-(4-methylphenyl)-5-phenylhydantoin (internal standard, IS) was purchased from Sigma-Aldrich (St. Louis, MO). HPLC-MS grade acetonitrile was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other common chemicals used were of the highest purity commercially available. Ultra-pure water from the Milli-Q ultra-pure system (Komatsu Electronics Co., Ltd., Ishikawa, Japan) was used in all experiments. Monolithic C_{18} Spin Tips (C_{18}-bonded monolithic silica gel with a diameter of 2.8 mm, thickness of 1 mm, weight of 2.5 mg, mesopore size of 10 nm, through pore size of 5 μm, and surface area of 350 m^2/g) were purchased from GL Sciences.

Preparation of plasma samples

Drug-free whole blood samples from healthy volunteers, who were recruited among laboratory personnel, were obtained intravenously in the presence of heparin sodium as an anticoagulant. To prepare drug-free plasma samples, heparinized whole blood was centrifuged at 1700 × *g* for 10 min at 4°C, and the plasma was decanted into a clean centrifuge tube. The drug-free plasma samples were stored at −80°C until use.

Preparation of standard solutions and quality control samples

Individual stock standard solutions (1 mg/ml) of the five barbiturates and the IS were prepared separately by dissolving an accurately weighed quantity of each drug in methanol. The solutions were then stored at 4°C. Working standard solutions of these drugs were prepared by appropriate dilution of the stock standard solutions using the HPLC mobile phase (10 mM ammonium acetate in 50% acetonitrile). All working standard solutions were freshly prepared every week and stored at 4°C. Calibration standards were prepared by mixing appropriate amounts of the working standard solutions and drug-free plasma to achieve seven different concentrations ranging from 5 to 500 ng/20 µl (5, 10, 25, 50, 100, 250, and 500 ng/20 µl) for five barbiturates, and 100 ng/20 µl for the IS. Quality

control (QC) samples (10–500 ng/20 µl) for all test drugs were also prepared using the same procedure.

Extraction procedure using Monolithic C_{18} Spin Tip

Extraction of five barbiturates and IS from human plasma and urine were achieved using a Monolithic C_{18} Spin Tip. Monolithic C_{18} Spin Tips were conditioned with 100 µl methanol and centrifuged at 1000 × *g* for 15 s, followed by 100 µl of ultra-pure water at 1000 × *g* for 15 s. For new tips, this procedure was repeated to reduce the background noise. To 20 µl of a human plasma sample containing 10 µl of drug mixture (five barbiturates and IS), 10 µl of 1 N HCl solution and 160 µl of ultra-pure water were added. For human urine samples of 100 µl of urine containing 10 µl of drug mixture (five barbiturates and IS), 10 µl of 1 N HCl solution and 80 µl of ultra-pure water were added. The sample solution was applied to the conditioned Monolithic C_{18} Spin Tip. The spin tip was centrifuged at 1000 × *g* for 15 s. The spin tip was then washed with 100 µl of ultra-pure water at 1000 × *g* for 15 s. After washing, the analytes were eluted from the spin tip with 50 µl of methanol at 1000 × *g* for 10 s. A 5-µl aliquot of the eluate was directly analyzed by UPLC-Q-ToF-MS.

UPLC-Q-ToF-MS conditions

A UPLC-Q-ToF-MS system consisting of an Acquity UPLC liquid chromatograph (Waters, Milford, MA, USA) and a Xevo G2 Q-TOF mass spectrometer (Waters, Milford, MA, USA) was used for all measurements.

The Waters Acquity UPLC system was equipped with a binary solvent manager, sample manager, and column oven. The chromatographic separation of the five barbiturates and IS was achieved on a Waters Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm., particle size 17-μm) with a linear gradient elution system composed of 10 mM ammonium acetate (pH 6.8) and methanol at a flow rate of 0.4 ml/min. Solvent A was ultra-pure water containing 10 mM ammonium acetate, and solvent B was methanol. Gradient runs were programmed to change from 90% solvent A/10% solvent B to 50% solvent A/50% solvent B within 2.5 min, and then to 1% solvent A/99% solvent B within 2.5 min. The column was subsequently maintained with 1% solvent A/99% solvent B for 1.0 min and then re-equilibrated with 90% solvent A/10% solvent B for 2.0 min before the next injection. The total chromatographic run time was 8 min.

For confirmation, mass spectrometric measurements were performed using a Xevo G2 Q-TOF mass spectrometer. The analyses were carried out using the ESI setting in the negative mode (ESI^-). The mass spectrometer was operated in negative ionization mode, with a capillary voltage of 2.8 kV and a cone voltage of 30 V. The source and desolvation temperatures

were 150°C and 500°C, respectively. The desolvation gas flow was 1000 l/hr and the cone gas flow 50 l/hr (both N_2). The mass range considered was 100–1000 Da. Data were collected in centroid mode, and the sensitivity analyzer mode was selected. The accuracy and reproducibility of all the analyses were guaranteed by the use of a LockSpray. Leucine-enkephalin was used as the lock mass at a concentration of 1 ng/ml in 50% of ultra-pure water-acetonitrile with 0.1% formic acid and a flow rate of 5 μl/min. MassLynx version 4.1 (Waters, Milford, MA, USA) was used to analyze the samples. The parameter settings were as follows: the analysis time was 0–6 min; the spectrum was above the relative intensity of 2%; the maximum tolerance of mass error was set as 5 ppm. The prediction rules of elemental composition (EC) were defined as follows: atom numbers of carbon, hydrogen, oxygen, nitrogen, and sulfur were set to ranges of 0–100, 0–200, 0–20, 0–20, and 0–6, respectively. The molecular formula assignments were obtained with the MassLynx i-Fit algorithm. For barbiturates and IS, the search was restricted to molecules containing CHONS only and the best Fit was obtained on both mass accuracy and isotope intensity pattern (i-Fit). Blank human plasma or urine samples were used as controls for comparison with the analytic samples, and all were processed under the same conditions.

Methods validation

The method was validated for linearity, selectivity, precision, accuracy, and recovery according to the US Food and Drug Administration guidelines for bioanalytical method validation [22]. Regression equations of the barbiturates were obtained by plotting the peak-area ratio of analytes/IS (y-axis) against the analyte concentration (x-axis). The slope and y-intercept of the regression line were estimated in duplicate for each of ten different calibrations and on six consecutive days. The acceptance criterion for the correlation coefficient was > 0.998. The limit of detection (LOD) was defined as the lowest concentration of analyte spiked in plasma that could be detected with a signal-to-noise ratio of at least 3. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve that could be measured with a signal-to-noise ratio of at least 10.

The selectivity of the method was estimated by analyzing blank human plasma matrix samples. The responses of the interfering substances or background noises at the retention time of barbiturates and IS were acceptable if they were less than 5% of the mean response of the LLOQ. Intra-day precision and accuracy were carried out by analyzing QC samples spiked with the five barbiturates at three different concentrations (10, 100, and 500 ng/20 µl) in six replicate samples on the same day. The

concentration of analytes in the QC samples was calculated using the calibration curves. The precision was determined by calculating the coefficient of variation (CV), while the accuracy was expressed as a percentage of the mean of measured concentration against the nominal concentration. The evaluations of precision were based on previously published criteria [22]. The acceptance criterion for precision (percentage CV) was ≤15%. Recovery was calculated by comparing the chromatographic peak areas of the analyte in QC samples with those obtained by direct injection of analyte standards dissolved in 10 mM ammonium acetate, and determined at different concentration levels (10, 100, and 500 ng/20 µl).

Administration of amobarbital to healthy volunteer

The present method was applied to real samples of human plasma to confirm its utility. A therapeutic dose of amobarbital (300 mg) was administered orally to a 40-year-old male volunteer (body weight, 73 kg). Informed consent was obtained from the subject. Whole blood samples were collected pre-dose (0 h) and 1.5 h after drug administration, and transferred to centrifuge tubes containing heparin sodium. The heparinized blood samples were centrifuged at 1700 × *g* for 10 min at 4 °C and plasma

samples were stored at −80 °C until analysis. This study was approved by the Ethics Committee of Showa University School of Medicine (No 860).

Results

The exracted ion chromatogram (XIC) chromatograms and mass spectral data for the five barbiturates and IS obtained by UPLC-Q-ToF-MS using an ESI are shown in Fig. 2 and Table 1. Phenobarbital, cyclobarbital, amobarbital, secobarbital, thiopental, and the IS detected in negative-ion mode gave the deprotonated molecule $[M-H]^-$ at m/z 231.0765, 235.1076, 225.1231, 2371232, 241.1006, and 265.0970, respectively, in the full-scan mode. These deprotonated molecules were identified as $C_{12}H_{11}N_2O_3$ (−2.2 ppm mass error, 0.020 i-Fit) for phenobarbital, $C_{12}H_{15}N_2O_3$ (−3.0 ppm mass error, 0.015 i-Fit) for cyclobarbital, $C_{11}H_{17}N_2O_3$ (−3.6 ppm mass error, 0.093 i-Fit) for amobarbital, $C_{12}H_{17}N_2O_3$ (−3.0 ppm mass error, 0.010 i-Fit) for secobarbital, $C_{11}H_{17}N_2O_2S$ (−2.1 ppm mass error, 0.461 i-Fit) for thiopental, and $C_{16}H_{13}N_2O_2$ (−1.6 ppm mass error, 0.017 i-Fit) for IS, respectively.

The regression equations of the five barbiturates showed good linearity from 5 to 500 ng/20 µl with correlation coefficients of at least 0.9984

(Table 2). The LOD and LLOQ of the barbiturates under optimal conditions were 1 ng/20 µl and 5 ng/20 µl for plasma, respectively (Table 2). Intra-day precisions and accuracies were evaluated by assessing QC samples prepared from human plasma (Table 3). The intra-day CVs were no greater than 7.8%, and the accuracies ranged from 86.3% to 97.9% for all concentrations. The recoveries of the five barbiturates from human plasma samples determined at three different concentrations ranged within 89.6–97.1% (Table 4).

In addition to analysis of spiked human plasma, the present method was applied to human plasma and urine samples collected following oral administration of amobarbital (300 mg) to a male volunteer. The amount of the IS was 100 ng/20 µl for plasma and 100 ng/100 µl for urine, respectively. Figure 3 shows UPLC-Q-ToF-MS XICs chromatograms and typical spectra of EC of human plasma 1.5 h after oral administration. The drug concentration in plasma calculated by internal calibration was 44.1 ng/20 µl 1.5 h after administration of amobarbital. The amobarbital and its hydroxide metabolite were identified using the present method from human urine 8 h after oral administration (Fig. 4). The deprotonated molecule was assigned as $C_{11}H_{17}N_2O_4$ (−2.5 ppm mass error, 0.354 i-Fit) for 3-hydroxyamobarbital (Fig. 4).

Discussion

Optimization of extraction conditions for Monolithic C_{18} Spin Tip

The entire Monolithic C_{18} Spin Tips extraction process, including conditioning, sample loading, washing, and elution required approximately 2 min. In contrast, the time required to manually perform conventional cartridge SPE was reported to be >20 min [23–26]. The eluate from the Monolithic C_{18} Spin Tips was directly injected into the UPLC-Q-ToF-MS without evaporation and reconstitution steps, which is particularly important for rapid and simple analysis. Therefore, the use of Monolithic C_{18} Spin Tips is recommended for rapid extraction of barbiturates and its metabolites from human body fluids. The total solvent volume used for each step of the extraction process was 500 µl, which is lower than volumes required by conventional SPE cartridges (around 5.6–65 ml) [23–26]. Furthermore, the required plasma or urine sample volume was reduced to 20 or 100 µl, respectively, which corresponds to 4.5–50 times less than volumes previously reported for barbiturates analysis in plasma and urine samples [23–26]. Thus, the small volumes of solvent and samples needed with the method used in this study represent a significant advance in sample preparation miniaturization and the overall procedure can be

considered to be “green” because it requires little solvent and produces little waste.

Method performance

Figure 2 shows TIC and XIC chromatograms obtained by UPLC-Q-ToF-MS for barbiturate drugs from human plasma containing the test compounds (50 ng per 20 µl). Distinct peaks appeared for each drug and the IS on the chromatograms within 4 min. Blank chromatograms gave small impurity peaks and no interfering peaks appeared around the retention times of the test compounds (data not shown). The spectra obtained showed the accurate corresponding masses for the deprotonated molecular ions. The criteria of the potential candidate ions used for EC analysis included mass error (mDa and ppm), double bond equivalent (DBE, total number of rings and double bonds in a molecule), empirical formula, and i-Fit value (the likelihood that the isotopic pattern of the EC matches a cluster of peaks in the spectrum). The potential calculated masses, mass accuracy, DBE, i-Fit value, and ECs associated with the measured mass of the marker ion were generated and analyzed with the ChemSpider and SciFinder libraries. The lower i-Fit value and the better the Fit Conf for barbiturates and IS were obtained (Table 1).

The present method showed good linearity for the known components tested, with LOD and LLOQ for human plasma under optimal conditions corresponding to 1 ng/20 µl and 5 ng/20 µl, respectively. Furthermore, lower i-Fit value was obtained under LLOQ concentration (Table 2). With therapeutic levels in plasma or serum of barbiturates reported to be 10000−40000 ng/ml for phenobarbital [27], 2000−6000 ng/ml for cyclobarbital [28], 2500−4000 ng/ml for amobarbital [29], 1000−2200 ng/ml for secobarbital [30], and 25000–50000 ng/ml for thiopental [31], the present method is sensitive enough to analyze these drugs at therapeutic levels.

The intra-day CVs at three concentrations examined were below 7.8%, and the accuracies ranged from 86.3% to 97.9% for all concentrations (Table 3), leading us to consider this variability acceptable for method validation based on current criteria [32, 33]. The recoveries of the five barbiturates from human plasma samples determined at three different concentrations ranged within 89.6–97.1% (Table 4). The reduced recovery (below 100%) is probably due to a loss of analyte during overall sample preparation steps. However, the reduction was not problematic, because satisfactory quantification was achieved using the method described, as shown in Table 2 and 3.

Actual measurements of amobarbital in human plasma after oral administration

In addition to analysis of spiked human plasma, the present method was applied to human plasma samples collected following oral administration of amobarbital to a male volunteer. Typical XIC chromatograms from this analysis are shown in Fig. 3. The drug concentration in plasma calculated by internal calibration was 44.1 ng/20 µl 1.5 h after administration of amobarbital. This concentration was within therapeutic levels and UPLC-Q-ToF-MS analysis [29].

Conclusions

We have established a detailed procedure for the quantitative determination and identification of barbiturates and a metabolite in human body samples via a novel Monolithic C_{18} Spin Tip and UPLC-Q-ToF-MS analysis. The use of Monolithic C_{18} Spin Tips represents an ideal sample preparation technique because of the simple extraction, and minimal sample and solvent requirements. The recoveries of the five drugs in plasma were 86−98% and the LLOQ was 5 ng/20 µl. The intra-day CVs for all the drugs in plasma were less than 7.8%. This method will be useful for

high-throughput determination of barbiturates in clinical and toxicological analyses. We are currently developing this technique for the detection of other classes of drugs in human body fluid samples.

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Conflict of interest disclosure

The authors declare no conflict of interest associated with this manuscript.

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Figure captions

Fig. 1. Appearance of a Monolithic C_{18} gel-packed Spin Tip and electron micrograph of the monolithic silica gel.

Fig. 2. TIC and XIC chromatograms of UPLC-Q-TOF-MS for barbiturate drugs from human plasma in negative ESI mode. The mixture of drugs (50 ng each) and 5-(4-methylphenyl)-5-phenylhydantoin used as IS (100 ng) were spiked into 20 µl of plasma. The MS spectra of XIC chromatograms are consistent with those reported in Table 1.

Fig. 3. UPLC-Q-Tof-MS XICs chromatograms and spectra of elemental composition from human plasma 1.5 h after oral administration of amobarbital (300 mg). The amount 5-(4-methylphenyl)-5-phenylhydantoin used as IS was 100 ng/20 µl of plasma.

Fig. 4. UPLC-Q-Tof-MS XICs chromatograms and spectra of elemental composition from human urine 8 h after oral administration of amobarbital (300 mg). The amount 5-(4-methylphenyl)-5-phenylhydantoin used as IS was 100 ng/100 µl of urine.

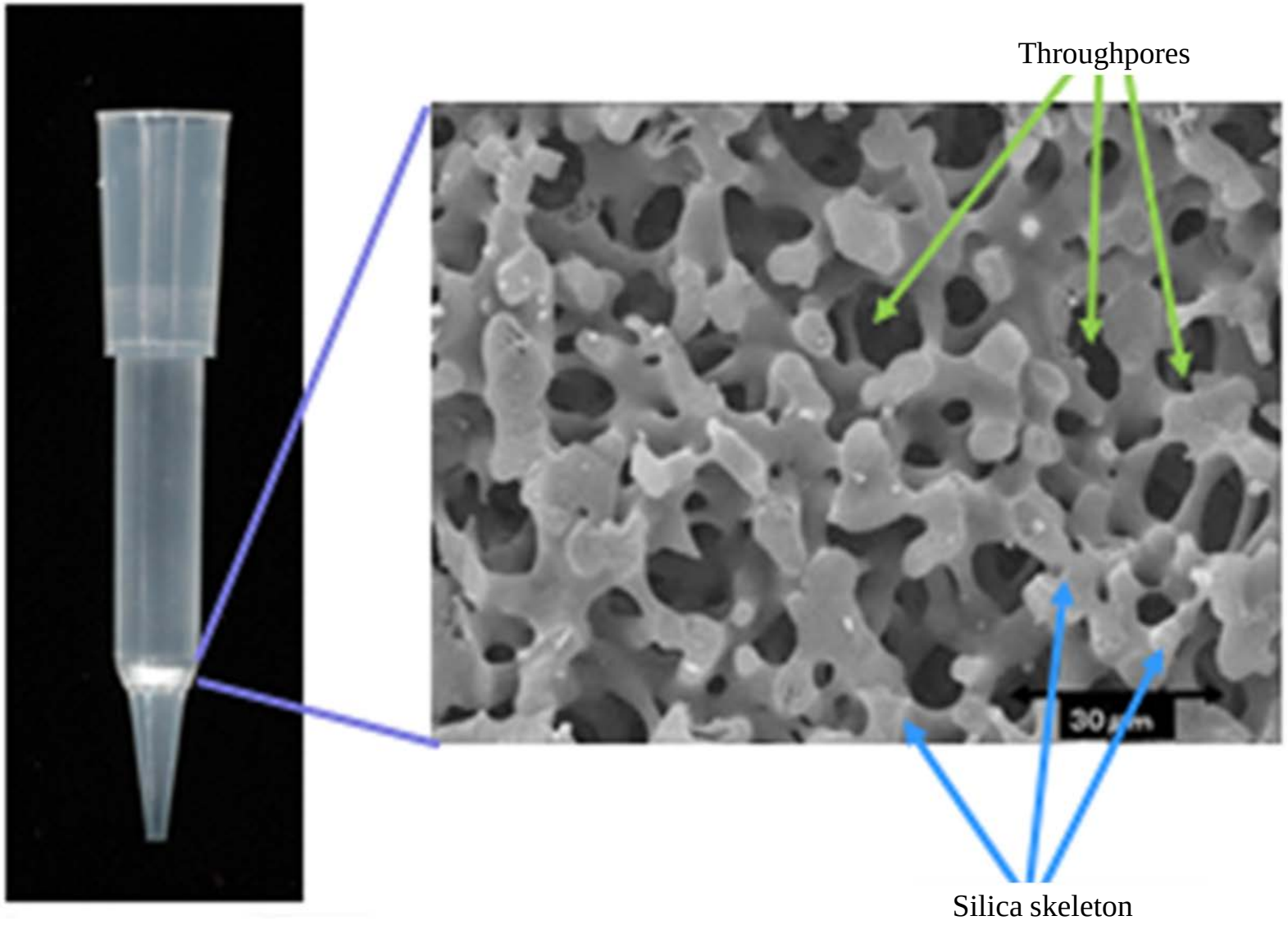


Fig. 1 Shouji et al

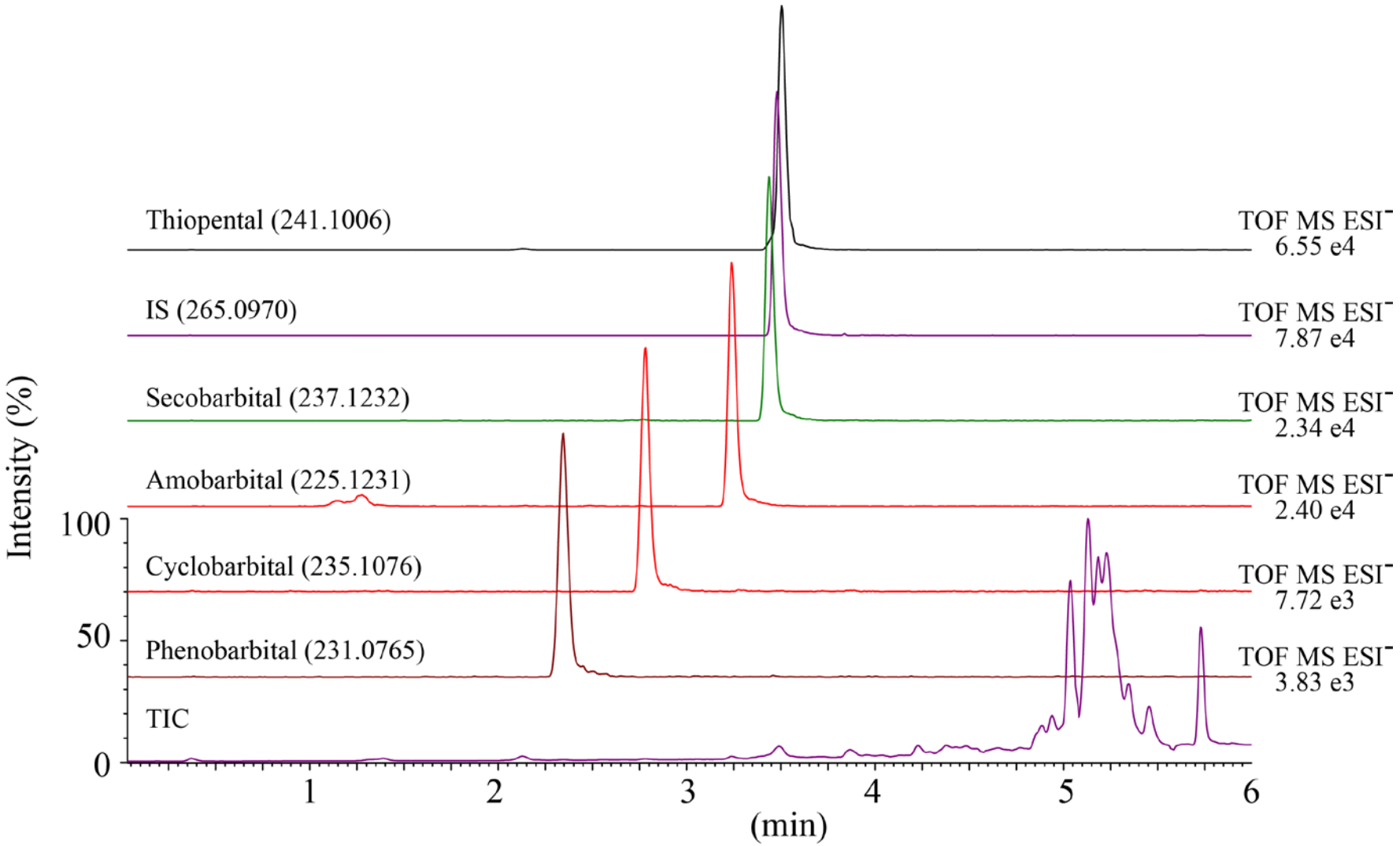
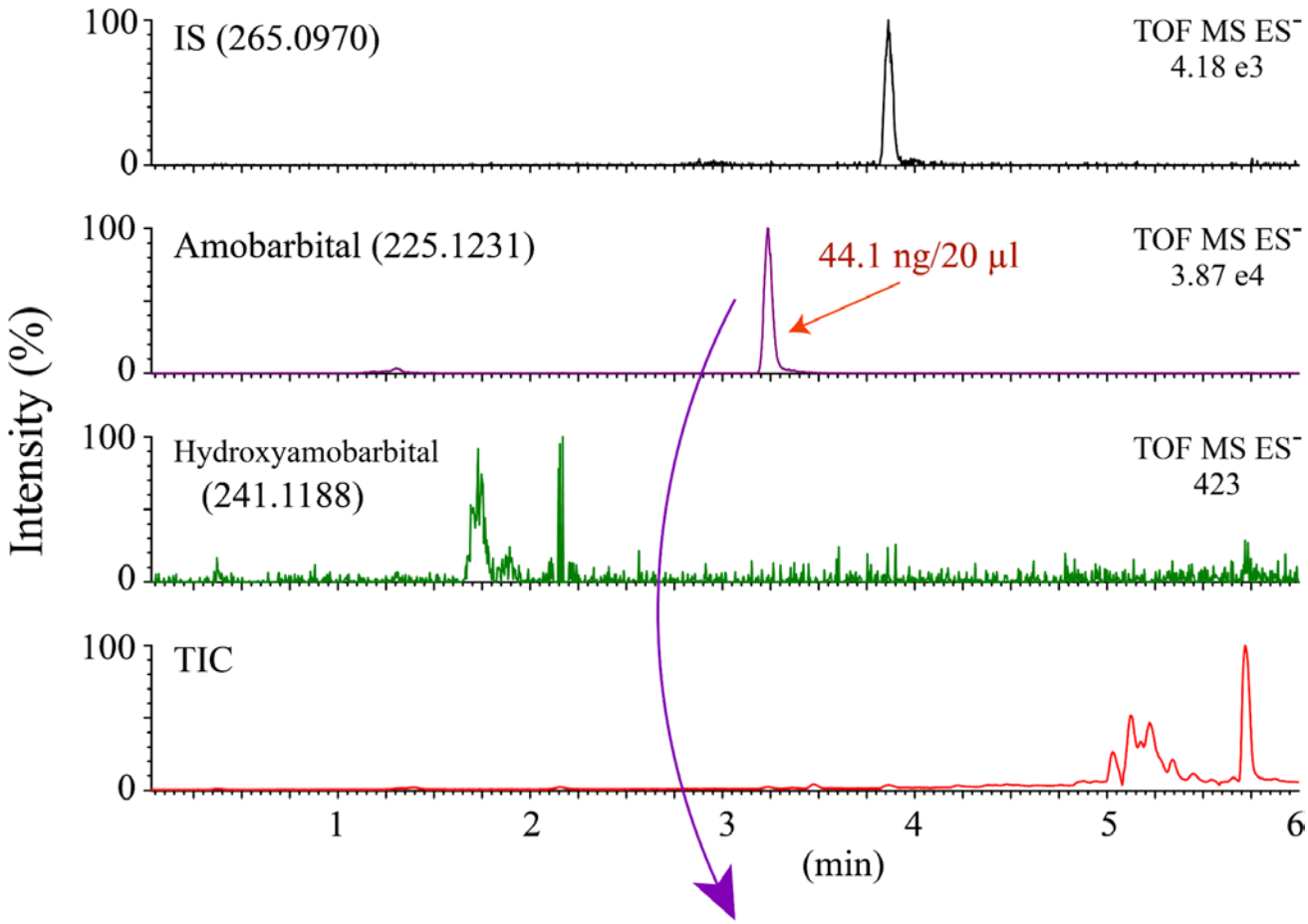


Fig. 2 Shouji et al



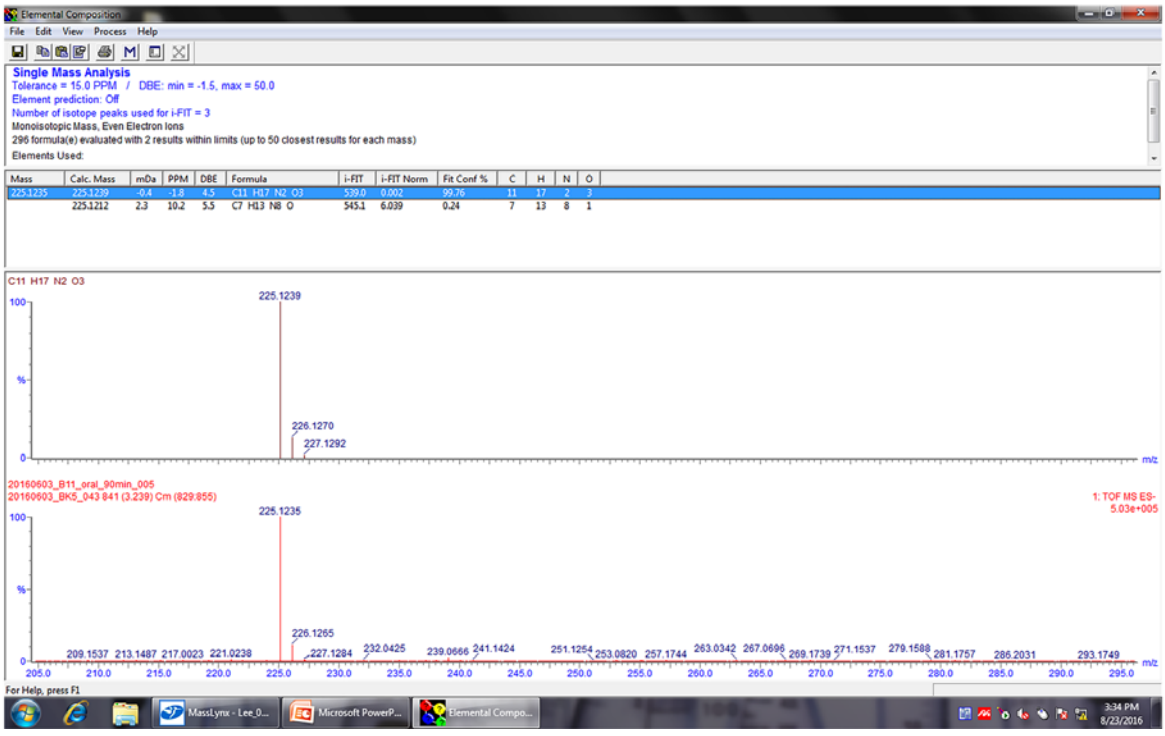
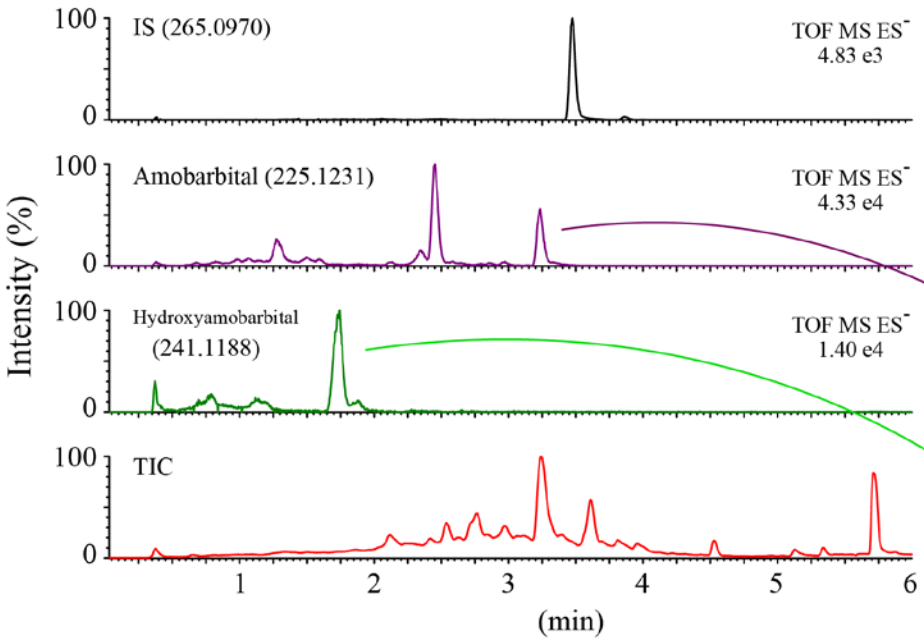
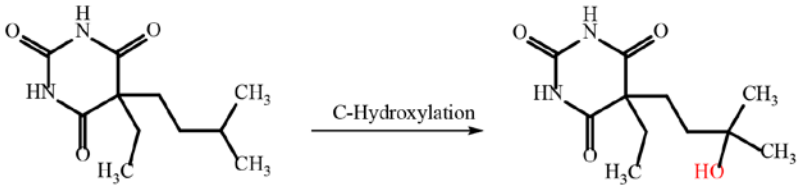


Fig. 3 Shouji et al





Metabolic pathway of amobarbital in human

Fig. 4 Shouji et al